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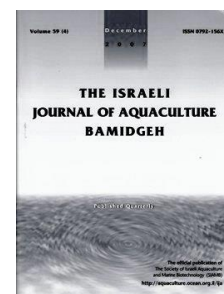
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## Effects of Trehalose, Bovine Serum Albumin, and Sucrose, on the Integrity of the Plasma Membrane of *Pseudosciaena crocea* Semen after Cryopreservation

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Key words: trehalose, bovine serum albumin, sucrose, *Pseudosciaena crocea*, cryopreservation

### Abstract

Cryopreservation and the effect of additives on the plasma membrane stability, motility, enzyme activity, membrane lipid composition, and ultrastructural injury of sperm of *Pseudosciaena crocea*, were examined in this study. Results indicated that the addition of trehalose (TH) and bovine serum albumin (BSA) provided significantly better results in the motility test compared to sucrose. Some BSA groups, showed significantly increased semen motility and effective enzyme activity. There were no significant differences between the cholesterol (CHO) composition for fresh sperm and cryopreserved sperm stored in 3 g/L BSA or 8.56 g/L TH+3 g/L BSA. Ultrastructural injury included damaged membrane, lost mitochondria, or broken flagella, whereas the majority of the freeze-thawed sperm remained morphologically normal. In conclusion, the addition of 3 g/L BSA and 8.56 g/L TH+3 g/L BSA significantly increased semen quality.

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## Introduction

Large yellow croaker, *Pseudosciaena crocea*, is a commercial marine species, is important in the aquaculture industry in south eastern China and elsewhere. However, over-fishing and water pollution have contributed to the decreasing numbers of wild populations (Lin et al., 1992). Reliable methods for sperm cryopreservation could benefit species conservation and improve breeding of this species.

Cryopreservation has been introduced as a favourable reproductive tool for the conservation of genetic resources through sperm banks. The evaluation of sperm membrane may reflect sperm quality and indicate the success of cryopreservation since the membranes are extremely susceptible to cryoinjury (Rurangwa et al., 2004; Li et al., 2006). Motility has been the most common factor examined for several species. In addition, the assessment of sperm motility combined with ultrastructural injury observation, are classical methods for detecting sperm damage (Yao et al., 2000). Both the cryopreservation of *P. crocea* sperm and the damage incurred during the process of cryopreservation have been previously reported (Lin and You 2002). Glycerol was used as a cryoprotectant for the successful storage of *P. crocea* sperm (Lin and You 2002). Subsequently, the effects of the freeze-thaw process on the sperm ultrastructure of *P. crocea* were examined (Lin et al. (2006). However, there is no exhaustive description of procedures for sperm motility evaluation, combined with enzyme activity assessment, membrane lipid composition detection, and ultrastructural injury observation.

Cryopreservation causes damage to cellular structures, which in turn can result in intracellular enzyme system dysfunction. To a certain extent, changes in antioxidant enzyme (i.e., superoxide dismutase (SOD) and catalase (CAT)), metabolism-related enzyme (i.e., adenosine triphosphate (ATP), lactate dehydrogenase (LDH), and succinate dehydrogenase (SDH)) and malondialdehyde (MDA) activity can reflect the physical activity of cells under different environmental conditions. Enzyme activity assessment has the advantage of being both simple and quick. Successful cryopreservation of sperm from mammals (Baumber et al., 2000; Cerolini et al., 2001; Tuncer et al., 2010), insects (Collins et al., 2004) and fish (Babiak et al., 2001; Butts et al., 2010; Yan and Zhang, 2011; Huang et al., 2012) has been achieved. Loss of sperm membrane lipids has a profound influence on the sperm membrane (Beirão et al. (2012). However, determination of sperm membrane lipid composition has been mainly limited to mammals (Baumber et al., 2000; Cerolini et al., 2001; Brinsko et al., 2007). Determining membrane lipid composition (cholesterol and phospholipids values), as suggested in the present study, can be used to conveniently and precisely assess sperm plasma membrane damage.

The plasma membrane is a key component of sperm and must be maintained throughout cryopreservation, if the sperm is to be kept alive. One of the easiest ways to test the success of a sperm cryopreservation protocol is to analyse cell membrane integrity. In addition, the quality of sperm can be assessed by viability tests, enzyme activity assessment, membrane lipid composition determination, and ultrastructural injury observation. These techniques can be decisive for the selection of good sperm samples and for standardization of the existing cryopreservation protocols.

Trehalose and sucrose probably play a key role in preventing deleterious alteration to the membrane during cryopreservation. Bovine serum albumin (BSA) is a membrane-stabilizing compound, which can protect the sperm's plasma membrane by enhancing membrane fluidity. These additives usually have a positive effect probably due to significant interactions between them (Tekin et al., 2007; Ciereszko et al., 2014; Lim et al., 2013). The effects of these three materials have been studied (Storey et al., 1998; Pérez-Pé et al., 2001; Aisen et al., 2002; Zhang et al., 2006; Varisli et al., 2013), but their effects on the integrity of the plasma membrane of *P. crocea* semen after cryopreservation have not been discussed. To the best of our knowledge, no studies have yet investigated the influence of these three materials on the viability of frozen-thawed *P. crocea*.

This study used viability tests, enzyme activity assessment, membrane lipid composition determination, and ultrastructural injury observation to evaluate the effects of supplementation with trehalose, BSA, sucrose, and their interactive groups on the stability of the plasma membrane of freeze-thawed *P. crocea* sperm. Our findings should help to optimize the additive types and concentrations for the cryopreservation of *P. crocea* sperm and provide a reliable method for cryopreserving and assessing sperm quality.

### Materials and Methods

Our research work was carried out in strict accordance with the requirements set out by the 'Governing Regulation for the Use of Experimental Animals in Zhejiang Province' (Zhejiang Provincial Government Order No. 263). None of the experimental fish were killed in this study.

**Samples.** Ten male fish (500–650 g) were collected from Haiwang Hatchery, Xiangshan, Zhejiang Province, in March 2013. DMSO, glutaraldehyde, trehalose, BSA, and sucrose, were purchased from the Nanking Jiancheng Biological Engineering Research Institute in China. Enzyme activity assay kits and lipids kits were purchased from the same company.

**Sperm cryopreservation.** Fresh sperm was collected from ten anesthetized male fish. The initial male ejaculate was discarded, and the external urogenital pore was wiped dry with a paper towel to avoid contamination by seawater, urine, and feces. To avoid differences in the quality of the sperm from different batches, only sperm with motility of >90% was used. One volume of sperm was mixed with three volumes of the extender, containing Cortland solution (7.25 g/L NaCl, 0.38 g/L KCl, 0.18 g/L CaCl<sub>2</sub>, 1.00 g/L NaHCO<sub>3</sub>, 0.23 g/L MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.41g/L NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O, 1.00 g/L C<sub>6</sub>H<sub>12</sub>O<sub>6</sub>, pH 7.00), 10% dimethyl sulfoxide (DMSO), and the following additives:

- TH1, 0.5 g/L trehalose;
- TH2, 8.56 g/L trehalose;
- TH3, 17.12 g/L trehalose;
- BSA1, 1 g/L bovine serum albumin;
- BSA2, 2 g/L bovine serum albumin;
- BSA3, 3 g/L bovine serum albumin;
- Suc1, 17.12 g/L sucrose;
- Suc2, 34.23 g/L sucrose;
- Suc3, 51.35 g/L sucrose;
- TH1+BSA1, 0.5 g/L trehalose+1 g/L bovine serum albumin;
- TH1+BSA2, 0.5 g/L trehalose+2 g/L bovine serum albumin;
- TH1+BSA3, 0.5 g/L trehalose+3 g/L bovine serum albumin;
- TH2+BSA1, 8.56 g/L trehalose+1 g/L bovine serum albumin;
- TH2+BSA2, 8.56 g/L trehalose+2 g/L bovine serum albumin;
- TH2+BSA3, 8.56 g/L trehalose+3 g/L bovine serum albumin;
- TH3+BSA1, 17.12 g/L trehalose+1 g/L bovine serum albumin;
- TH3+BSA2, 17.12 g/L trehalose+2 g/L bovine serum albumin;
- TH3+BSA3, 17.12 g/L trehalose+3 g/L bovine serum albumin.

Based on the procedure of Bozkurt et al. (2014), sperm samples were kept on ice for 5 min and then collected into 250 µL straws. The straws were placed horizontally 3 cm above the surface of liquid nitrogen. After a freezing period of 3 min, the straws were plugged into nitrogen for storage following the procedure described by Tiersch et al. (1994) and Ye et al. (2009). Each treatment was repeated ten times.

**Determination of sperm motility.** The motility of fresh sperm and cryopreserved sperm was monitored for 7 days, following the procedure described by Tiersch et al. (1994) and Lin and You (2002). Motility was determined under a dark-field microscope. Thawed samples were diluted in the cryopreservation extender. Two microliters of sperm were placed on a microscope slide and diluted with filtered seawater. The sperm became motile and initiated rapid swimming. The percentage of sperm swimming actively in a forward direction (activation rate), time until in situ fibrillation (movement time), and

time until complete cessation of motility (lifespan) were estimated at 200× magnification. The activation rate was estimated at 10 s intervals. Sperm motility was evaluated subjectively in triplicate by the same observer.

**Determination of enzyme activity.** Assays were performed in sperm samples immediately after thawing. Enzymes were extracted using the procedure described by Kankofer et al. (2005) and Zhang et al. (2009). Frozen semen samples were thawed in a water bath at 40 °C and gently homogenized and centrifuged (3,000 r/min) for 15 min at 4°C. Subsequently, the sperm pellet was separated from the supernatant and washed with normal saline (NS) three times. After centrifugation, the pellet was incubated with NS for 20 min at 4°C. The supernatant was collected by centrifugation at 3,000 r/min for 10 min and kept at 4°C until enzyme determination. Enzyme concentrations were analyzed using a kit-based spectrophotometric method (Nanking Jiancheng Biological Engineering Research Institute, China).

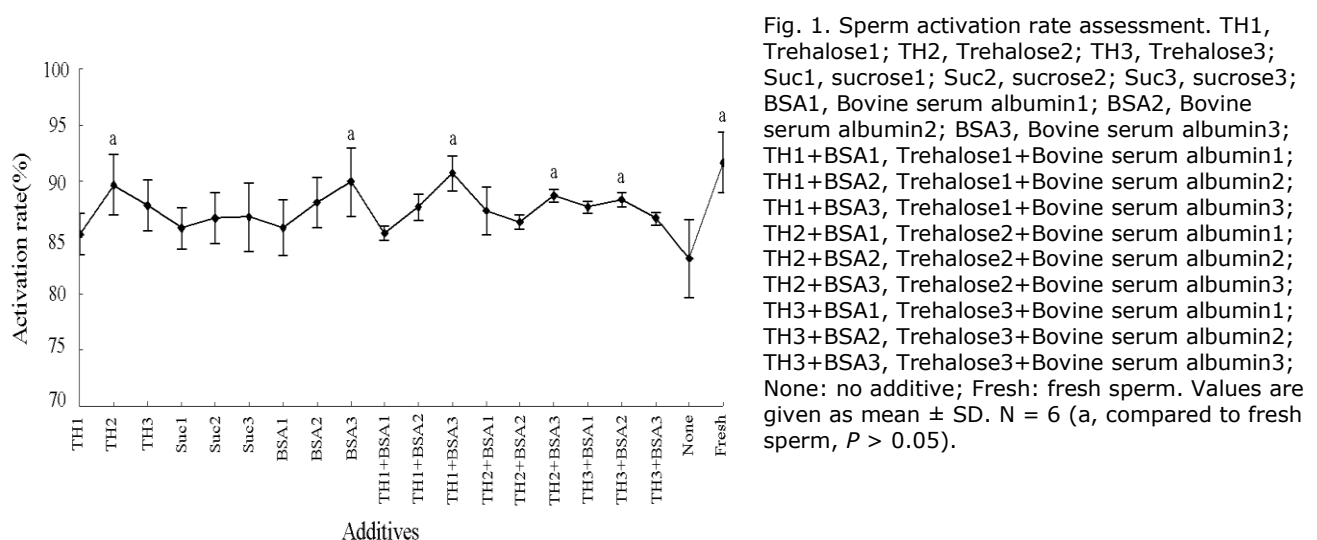
**Determination of membrane lipid composition (cholesterol and phospholipids).** Straws were thawed in a water bath at 40°C, and lipids were extracted based on the procedure of Zheng et al. (2008). Samples were kept at 4°C until lipid determination. Cholesterol and phospholipids were measured according to the manufacturer's instruction (Nanjing Jiancheng Bioengineering Institute).

**Determination of ultrastructure damage.** Fresh and freeze-thawed semen samples were fixed in 2.5% glutaraldehyde. After 5 h, the samples were dehydrated with graded ethanol, followed by four changes of *tert*-butyl alcohol (TBA). Semen were then critical-point dried and gold coated (Wu et al., 2007; Gwo, 2010). The ultrastructure of sperm was observed under a JSM-S3400N scanning electron microscope.

**Statistical analysis.** Analysis of variance was performed using SPSS for Windows 11.5; data are presented as mean ± SD. Motility, enzyme activity, and lipid concentration were analysed using one-way ANOVA. Results were considered significant at  $P < 0.05$ .

## Results

**Sperm motility.** We found no significant difference between some of the experimental groups and the control group (fresh sperm), in terms of activation rate, movement time, and lifespan. Those three indices in the other experimental groups were significantly lower than those for fresh sperm. The addition of sucrose did not have any significant effect on sperm motility and the freezing extender. TH2, BSA3, TH1+BSA3, TH2+BSA3, and TH3+BSA2 provided better results; in particular, BSA3 and TH2+BSA3 significantly improved sperm motility compared to the control group ( $P > 0.05$ ) (Figs. 1, 2).



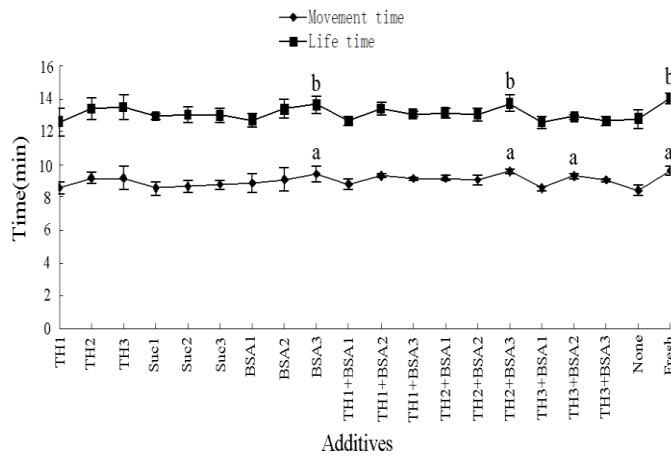


Fig. 2. Sperm movement time and life time assessment. TH1, Trehalose1; TH2, Trehalose2; TH3, Trehalose3; Suc1, sucrose1; Suc2, sucrose2; Suc3, sucrose3; BSA1, Bovine serum albumin1; BSA2, Bovine serum albumin2; BSA3, Bovine serum albumin3; TH1+BSA1, Trehalose1+Bovine serum albumin1; TH1+BSA2, Trehalose1+Bovine serum albumin2; TH1+BSA3, Trehalose1+Bovine serum albumin3; TH2+BSA1, Trehalose2+Bovine serum albumin1; TH2+BSA2, Trehalose2+Bovine serum albumin2; TH2+BSA3, Trehalose2+Bovine serum albumin3; TH3+BSA1, Trehalose3+Bovine serum albumin1; TH3+BSA2, Trehalose3+Bovine serum albumin2; TH3+BSA3, Trehalose3+Bovine serum albumin3; None: no additive; Fresh: fresh sperm. Values are given as mean  $\pm$  SD. N = 6. Values with the same letters are not significantly different

**Enzyme activity.** As shown in Figs. 3, 4, 5, 6, malondialdehyde(MDA) levels increased during cryopreservation, while SOD, CAT, ATP, LDH, and SDH activity was reduced. We found no significant difference between some of the experimental groups and the control group, in terms of enzyme activity ( $P > 0.05$ ). More specifically, TH2 led to higher SOD, CAT, and SDH activity; TH1+BSA3 led to higher SOD, CAT and LDH activity; TH2+BSA3 led to higher SOD, CAT, LDH and SDH activity; TH3+BSA2 led to higher SOD, CAT and LDH activity; TH3 and TH2+BSA1 led to higher CAT activity; BSA3 led to higher SOD activity; TH1+BSA2 led to higher SDH activity (Figs. 3, 4, 5, 6); and the other experimental groups showed significantly lower enzyme activity than that observed for fresh sperm. TH2, TH2+BSA3 and TH3+BSA2 led to lower MDA activity (Fig. 4); and the other experimental groups showed significantly higher enzyme activity than that observed for fresh sperm.

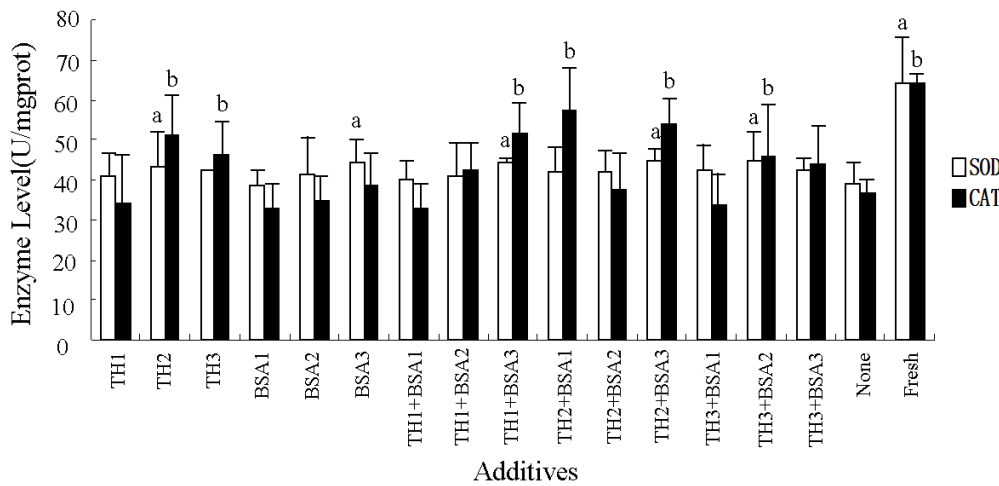


Fig. 3. Antioxidant enzyme (SOD, CAT) assessment. TH1, Trehalose1; TH2, Trehalose2; TH3, Trehalose3; BSA1, Bovine serum albumin1; BSA2, Bovine serum albumin2; BSA3, Bovine serum albumin3; TH1+BSA1, Trehalose1+ Bovine serum albumin1; TH1+BSA2, Trehalose1+Bovine serum albumin2; TH1+BSA3, Trehalose1+Bovine serum albumin3; TH2+BSA1, Trehalose2+Bovine serum albumin1; TH2+BSA2, Trehalose2+Bovine serum albumin2; TH2+BSA3, Trehalose2+Bovine serum albumin3; TH3+BSA1, Trehalose3+Bovine serum albumin1; TH3+BSA2, Trehalose3+Bovine serum albumin2; TH3+BSA3, Trehalose3+Bovine serum albumin3; None: no additive; Fresh: fresh sperm. Values are given as mean  $\pm$  SD. N = 6. Columns with the same letters are not significantly different ( $P > 0.05$ ).

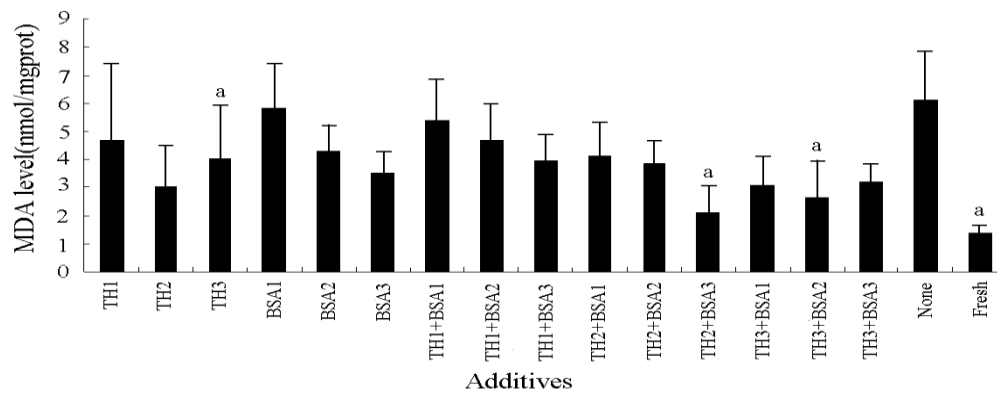


Fig. 4. MDA assessment. TH1, Trehalose1; TH2, Trehalose2; TH3, Trehalose3; BSA1, Bovine serum albumin1; BSA2, Bovine serum albumin2; BSA3, Bovine serum albumin3; TH1+BSA1, Trehalose1+Bovine serum albumin1; TH1+BSA2, Trehalose1+Bovine serum albumin2; TH1+BSA3, Trehalose1+Bovine serum albumin3; TH2+BSA1, Trehalose2+Bovine serum albumin1; TH2+BSA2, Trehalose2+Bovine serum albumin2; TH2+BSA3, Trehalose2+Bovine serum albumin3; TH3+BSA1, Trehalose3+Bovine serum albumin1; TH3+BSA2, Trehalose3+Bovine serum albumin2; TH3+BSA3, Trehalose3+Bovine serum albumin3; None: no additive; Fresh: fresh sperm. Values are given as mean  $\pm$  SD. N = 6. Columns with the same letters are not significantly different ( $P > 0.05$ ).

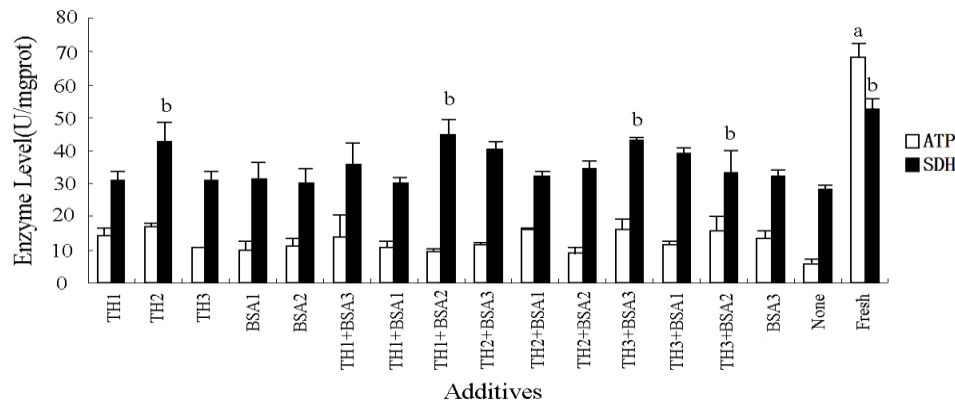


Fig. 5. ATP and SDH assessment. TH1, Trehalose1; TH2, Trehalose2; TH3, Trehalose3; BSA1, Bovine serum albumin1; BSA2, Bovine serum albumin2; BSA3, Bovine serum albumin3; TH1+BSA1, Trehalose1+Bovine serum albumin1; TH1+BSA2, Trehalose1+Bovine serum albumin2; TH1+BSA3, Trehalose1+Bovine serum albumin3; TH2+BSA1, Trehalose2+Bovine serum albumin1; TH2+BSA2, Trehalose2+Bovine serum albumin2; TH2+BSA3, Trehalose2+Bovine serum albumin3; TH3+BSA1, Trehalose3+Bovine serum albumin1; TH3+BSA2, Trehalose3+Bovine serum albumin2; TH3+BSA3, Trehalose3+Bovine serum albumin3; None: no additive; Fresh: fresh sperm. Values are given as mean  $\pm$  SD. N = 6. Columns with the same letters are not significantly different ( $P > 0.05$ ).

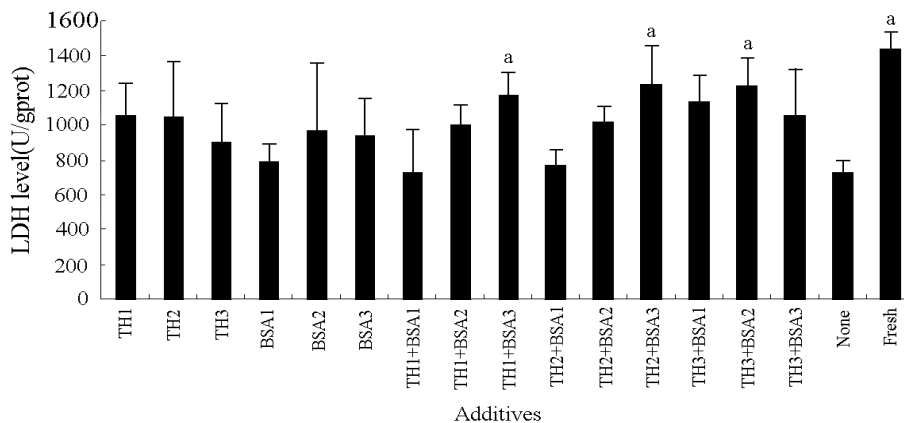


Fig. 6. LDH assessment. TH1, Trehalose1; TH2, Trehalose2; TH3, Trehalose3; BSA1, Bovine serum albumin1; BSA2, Bovine serum albumin2; BSA3, Bovine serum albumin3; TH1+BSA1, Trehalose1+Bovine serum albumin1; TH1+BSA2, Trehalose1+Bovine serum albumin2; TH1+BSA3, Trehalose1+Bovine serum albumin3; TH2+BSA1, Trehalose2+Bovine serum albumin1; TH2+BSA2, Trehalose2+Bovine serum albumin2; TH2+BSA3, Trehalose2+Bovine serum albumin3; TH3+BSA1, Trehalose3+Bovine serum albumin1; TH3+BSA2, Trehalose3+Bovine serum albumin2; TH3+BSA3, Trehalose3+Bovine serum albumin3; None: no additive; Fresh: fresh sperm. Values are given as mean  $\pm$  SD. N = 6. Columns with the same letters are not significantly different ( $P > 0.05$ ).



**Cholesterol and phospholipids composition.** The effect of trehalose on post-thaw sperm motility and recovery rate is summarized in Table 1. We found no significant difference between the experimental groups and the control group, in terms of cholesterol content ( $P > 0.05$ ), and we also found no significant difference among the BSA3 and TH2+BSA3 groups, and the control group, in terms of cholesterol content ( $P > 0.05$ ). Furthermore, cholesterol/phospholipids (c/p) values decreased during cryopreservation. The addition of TH2, TH3, BSA3, TH2+BSA2, or TH2+BSA3 yielded higher c/p values (higher than 0.50).

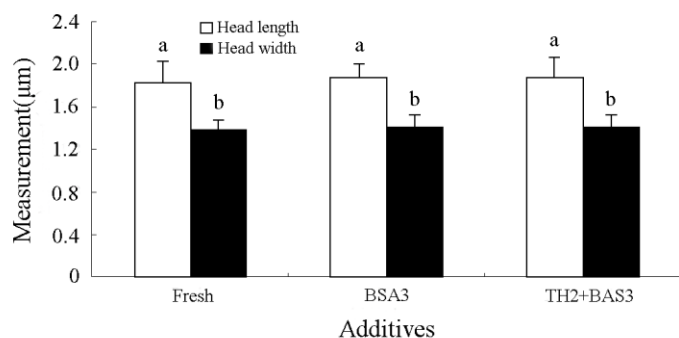
**Table 1.** Effect of additives on changes in lipids in *P. crocea* spermatozoa during freezing (nmol/ $10^9$  sperm)<sup>1</sup>

Additive	g/L	Abbreviation	Cholesterol	Phospholipids	cholesterol/phospholipids
Trehalose	0.5	TH1	113.16±30.00	241.25±37.55	0.47
Trehalose	8.56	TH2	134.72±28.51	271.21±14.45	0.50
Trehalose	17.12	TH3	129.33±9.33	260.18±20.62	0.50
Bovine serum albumin	1	BSA1	107.78±30.00	239.68±28.51	0.45
Bovine serum albumin	2	BSA2	114.96±18.92	250.71±45.13	0.46
Bovine serum albumin	3	BSA3	141.90±18.92 <sup>a</sup>	285.40±14.45	0.50
Trehalose+ bovine serum albumin	0.5+1	TH1+BSA1	100.59±16.46	249.14±42.40	0.40
Trehalose+ bovine serum albumin	0.5+2	TH1+BSA2	125.74±20.40	255.44±18.92	0.49
Trehalose+ bovine serum albumin	0.5+3	TH1+BSA3	141.90±11.22	291.71±17.71	0.49
Trehalose+ bovine serum albumin	8.56+1	TH2+BSA1	109.57±20.40	242.83±44.71	0.45
Trehalose+ bovine serum albumin	8.56+2	TH2+BSA2	131.13±8.23	263.33±31.50	0.50
Trehalose+ bovine serum albumin	8.56+3	TH2+BSA3	147.29±50.07 <sup>a</sup>	280.67±23.33	0.52
Trehalose+ bovine serum albumin	17.12+1	TH3+BSA1	140.11±16.17	288.56±14.19	0.49
Trehalose+ bovine serum albumin	17.12+2	TH3+BSA2	129.33±10.78	275.94±21.68	0.47
Trehalose+ bovine serum albumin	17.12+3	TH3+BSA3	122.15±18.92	274.37±29.54	0.45
No additive	/	None	111.37±13.56	245.98±12.52	0.45
Fresh sperm	/	Fresh	172.44±11.67 <sup>a</sup>	286.98±28.90	0.60

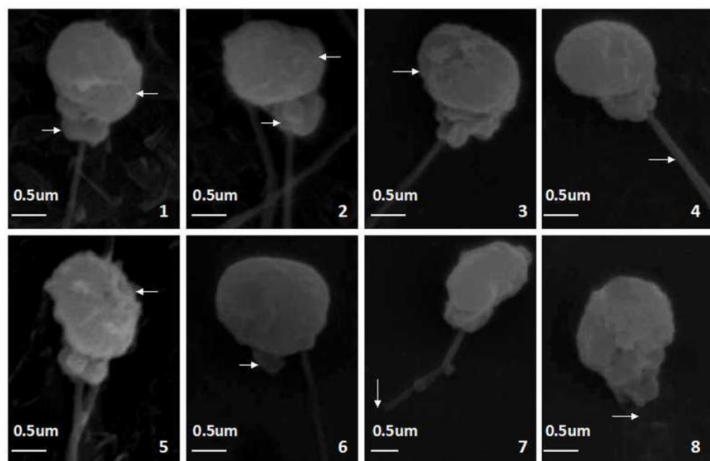
<sup>1</sup> Values are given as mean ± SD. N = 6 (<sup>a</sup> compared to fresh sperm,  $P > 0.05$ ). Where TH= Trehalose and BSA= Bovine serum albumin.

**Ultrastructure damage.** Based on the data from our above assessments, we chose BSA3 and TH2+BSA3 to assess ultrastructure damage (Figs. 7, 8; Table 2). As shown in Fig. 7, there was no significant difference between these groups and the control group (fresh sperm), in terms of the variation of the sperm's plasma membrane, and Fig. 8 and Table 2 show that, in both fresh and freeze-thawed sperm, membranes were damaged, mitochondria were lost or dilated, and tails were broken, with a non-significant proportion of damaged sperm; the majority of the freeze-thawed sperm remained morphologically normal.





**Fig. 7.** Sperm head measurement. This figure shows the effects of different additives on sperm head length and width of large yellow croaker sperm after scanning electron microscope assessment. Fresh: fresh sperm; BSA3, Bovine serum albumin3; TH2+BSA3, Trehalose2+ Bovine serum albumin3. Values are given as mean  $\pm$  SD. N = 100. Columns with the same letters are not significantly different ( $P > 0.05$ ).



**Fig. 8.** Scanning electron microscope assessment. 1. Normal sperm ( $\leftarrow$ showing the normal head and  $\rightarrow$ showing the normal mitochondria); 2. Normal sperm ( $\leftarrow$ showing the normal head and  $\rightarrow$ showing the normal mitochondria); 3. Normal sperm ( $\rightarrow$ showing the normal head); 4. Normal sperm ( $\rightarrow$ showing the normal flagellum); 5. Abnormal sperm ( $\leftarrow$ showing the abnormal head); 6. Abnormal sperm ( $\rightarrow$ showing the mitochondria lost); 7. Abnormal sperm ( $\downarrow$  showing the broken flagellum); 8. Abnormal sperm ( $\rightarrow$ showing the lost flagellum) (bar = 0.5  $\mu$ m).

**Table 2.** Data of scanning electron microscope examination (N=100)

Additive	Abnormal head (%)	Abnormal mitochondria (%)	Abnormal flagellum (%)	Abnormal sperm (%)
Fresh sperm	16	4	2	20
BSA3	18	4	1	21
TH2+BSA3	18	3	3	23

## Discussion

In previous tests trehalose had better cryoprotective effects than sucrose or Tris-citric acid-glucose (TCG) and it was hypothesized that the cryoprotective effects of trehalose were due to enhanced sperm membrane fluidity before freezing (Aboagla and Terada 2003). Yildiz et al. (2000) compared the influence of sugar supplementation of the extender on the motility, viability, and acrosomal integrity of dog spermatozoa during freezing and concluded that trehalose could significantly increase the total percentage of active sperm. Aisen et al. (2000) found similar results. In addition, it was found that trehalose and BSA significantly improved the quality of cryopreserved bull semen, with the interaction effects of trehalose (0.00037 mol/L)+BSA (2 g/L) being the best combination (Zan et al. 2008).

Several researchers have found that incorporating trehalose and BSA in sperm diluents significantly protected the spermatozoa of many species against freezing damage, in comparison to sucrose. In spite of this evidence in favor of the beneficial effects of trehalose and BSA, not all studies obtained the same results. Sucrose was better than trehalose in preserving the motility and acrosome integrity of bovine spermatozoa after freezing and thawing (Woelders et al. (1997). The usefulness of six sugars (sucrose, maltose, trehalose, galactose, fructose, and glucose) was tested at three concentrations (125, 250, and 500 mM) on trout semen and sucrose used at 125 mM was found to provide the most beneficial effects (Maisse (1994). We believe that the different results may be caused by different experimental material or methods. Semen characteristics vary among species, stocks, and even within samples from the same animal, depending on the collection period during the reproductive season. Moreover, freezing and thawing protocols play an important role in cryopreservation.

In the present study, we extrapolated that the effectiveness of spermatozoa with BSA3 before freezing could increase sperm membrane fluidity, rendering the spermatozoa capable of enduring freeze-thawing damage. The mechanism for the cryoprotective action of BSA and its modulation by BSA concentration needs to be examined in further studies.

Antioxidant enzymes (SOD and CAT) and metabolism-related enzymes (ATP, LDH, and SDH) activity of semen after cryopreservation have been assessed in fish such as *Monopterus albus* and *Acipenser gueldenstaedtii* (Yan and Zhang, 2011; Huang et al., 2014). Nevertheless, few studies have been conducted to investigate the effects of different additives on the enzyme and MDA activity in fish semen. Our work was performed to assess the activity of the above physiological indicators in *P. crocea* semen with the addition of TH and/or BSA; we concluded that the TH2, TH1+BSA3, TH2+BSA3, and TH3+BSA2 groups led to higher enzyme activity. Moreover, we found that BSA3 led to better motility, whereas it did not provide ideal results in terms of enzyme activity, with the exception of SOD. We suspect that low amounts of SDH, LDH, and ATP are mainly concentrated in the mitochondrial sheath. The mitochondrial damage induced in sperm is typical of freezing damage, which may lead to a reduction in enzymes. Minor differences in the levels of enzymes may result in greater variability. We could not conclude that all sperm assayed were fully developed, and this would affect the levels of metabolism-related enzymes. We also hypothesized that SOD detection might be more effective than that of other enzymes. BSA3 may be one of the most effective additives for cryopreservation of sperm. We also suggested that SOD should be sperm assayed and fully used to assess enzyme activity and the integrity of sperm plasma membrane.

Maintaining an appropriate lipid composition is important in teleost fish, since sperm needs to be resistant to fluctuations in osmolarity during the process of cryopreservation and fertilization. The efflux of cholesterol from the sperm plasma membrane and the resultant decrease in the cholesterol/phospholipid (c/p) molar ratio are important (Brinsko et al., 2007). The present study confirmed that most groups of additive-treated sperm had a significant reduction in cholesterol content, except for the BSA3 and TH2+BSA3 treatment groups. Phospholipid content, however, at the basic level, led to a decrease in the c/p value. Using this information, we can choose better cryopreservation additives. High levels of cholesterol resulted in a more cohesive, rigid, and impermeable structure (Cabrita et al. 2010). In general, spermatozoa that are more resistant to cold shock have a higher cholesterol/phospholipid molar ratio.

Sperm morphology and membrane ultrastructure are often evaluated in cryopreserved fish sperm using scanning electron microscopy (SEM) (Cabrita et al., 2010). Using SEM, we could identify morphological and morphometric changes in the head, mitochondria, and flagella of post-thawed sperm, which agree with the results of Zhang et al. (2003) and Ji et al. (2004). Because plasma membrane expansion is obvious in cryopreservation, we measured the head length, width of fresh sperm, and freeze-thawed sperm, and found no significant differences. On the other hand, when we used SEM, we discovered that the sperm had broken cell membranes, lost or dilated mitochondria, or broken flagella, although the majority of the freeze-thawed sperm remained morphologically normal. Thus, we speculate that BSA3 and TH2+BSA3 sperm groups had intact ultrastructure.

The sperm used in the present experiment were obtained from the same mature male fish, and each group of sperm was treated with the same freeze-thaw process. Effects of mechanical injury in sperm cryopreservation had no significant influence on sperm plasma membrane stability. Therefore the differing results of cryopreserved sperm were caused by additive types and concentration. The present study integrated our understanding of: 1) sperm motility, 2) enzyme activity, 3) cholesterol and phospholipids composition, and 4) ultrastructure damage observation of *P. crocea*; we obtained two better groups: BSA3 and TH2+BSA3. To make the results applicable, an in vivo test in an induced spawn of *P. crocea* is needed for future study.

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